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Tyrosine Kinase Inhibitors as Antiproliferative Agents Against an Estrogen-Dependent Breast Cancer Cell Line In Vitro

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Background and Objectives: Receptor tyrosine kinase (RTK) activation is critical for growth factor-mediated cell proliferation. Blockade of RTK activation inhibits growth factor-induced cell proliferation. A panel of RTK inhibitors (tyrphostins) have been tested and compared for their antiproliferative effects on the hormone-dependent human breast cancer cell line, MCF-7, in vitro.

Methods: MCF-7 cells (10^4/well) were seeded into 96 well plates and maintained in DMEM with 1% bovine serum albumin (BSA), 200-pg/mL estrogen, or 10% fetal bovine serum. After a defined time interval, the cells were exposed to RTK inhibitors and a non–RTK-inhibitory analog of tyrphostins (0 to 400 μM). After 3 days, the number of viable cells in each well was estimated by an MTT assay and the results expressed as percent of controls. Using a representative tyrphostin, A47, the validity of MTT assay as a measure of cell proliferation was tested by a colony formation assay and by immunostaining with Ki-67 antibodies.

Results: MCF-7 cells maintained in DMEM containing 1% BSA without E2 or serum showed a minimal increase in cell number. Supplementation with E2 stimulated cell proliferation in a dose-dependent manner. This E2-mediated growth stimulation was completely inhibited (cytostatic effects) by the epidermal growth factor receptor (EGFR)-selective tyrphostins A47, B48, RG13022, and B50. These same tyrphostins also decreased the cell numbers to below control numbers in cultures maintained in 1% BSA or in serum containing medium (cytostatic/cytotoxic effects). B44 (EGFR-selective tyrphostin), AG1295 (platelet-derived growth factor receptor [PDGFR]-selective tyrphostin), and A1 had no inhibitory effects on cells with or without E2 treatments. However, A1 inhibited cell growth under serum supplementation. Genistein, a phytoestrogen, stimulated the autonomous, E2-induced as well as serum-induced growth of MCF-7 cells. Cell proliferation results derived from the MTT assay were corroborated by both the colony formation assay as well as the Ki-67 assay.

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Conclusions: Of the agents tested, only EGFR-selective tyrphostins blocked E2-stimulated tumor cell proliferation, as opposed to the PDGFR-selective tyrphostin, RTK noninhibitory agent, or the phytoestrogen, genistein, which did not exert such an effect. These findings suggest that epidermal growth factor (EGF) is an important mediator of E2-induced proliferation of MCF-7 cells. Thus, tyrphostins may be selectively used to prevent the growth of hormone-dependent breast cancers, particularly regrowth of residual tumor in postmenopausal breast cancer survivors receiving estrogen replacement therapy.

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KEY WORDS: breast cancer; estrogen; growth factor receptors; tyrphostins; MCF-7 cell line

INTRODUCTION

Polypeptide growth factors have long been established as important mitogenic stimulants for normal as well as neoplastic cells [1,2]. Growth factor-mediated mitogenic stimulation begins following selective recognition and interaction of the growth factors with their respective cell surface receptors. Once the growth factors bind to their receptors, the receptors generally dimerize and undergo phosphorylation of their cytoplasmic tyrosine kinase domains. Activation of these receptor tyrosine kinases (RTKs) leads to further phosphorylation of a series of intracellular substrates and finally signals the cells to proliferate. In this pathway of signal transduction, phosphorylation of RTKs is essential, thereby making blockade of RTK activation an attractive strategy to prevent growth factor-induced proliferation of cancer cells.

In the past decade, a family of low-molecular-weight compounds, tyrphostins, have been synthesized and identified as potent inhibitors of RTKs [3–5]. Different members of the tyrphostin family recognize RTKs of different growth factor receptors such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGF-1R), and platelet-derived growth factor receptor (PDGFR) in a selective manner [3,4]. Accordingly, different tyrphostins selectively block growth-stimulatory effects of different growth factors. Tyrphostins that block growth factor RTKs have indeed been found to be effective inhibitors of tumor growth in vitro and in vivo [6–9].

We report on the antiproliferative effects of a panel of tyrphostins differing in their RTK selectivity and in vitro tyrosine kinase-inhibitory potencies on the hormone-dependent MCF-7 human breast cancer cell line. Utilization of this cell line has enabled us to determine the effects of tyrphostins on cell proliferation under different conditions such as in the absence of any added external mitogenic factor, proliferation induced by serum factors, as well as proliferation induced by estrogen. Our studies also compare the effectiveness of different tyrphostins on a single defined cell line. Furthermore, since the in vitro tyrosine kinase enzyme-inhibitory activities of these agents are known, it would provide an opportunity to determine whether these activities are predictive of the in vivo cell growth-inhibitory activities of these agents.

MATERIALS AND METHODS

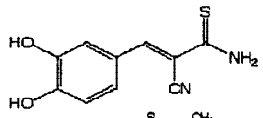
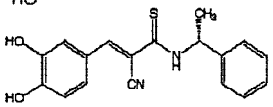
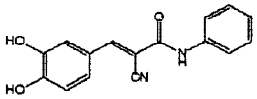
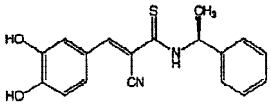
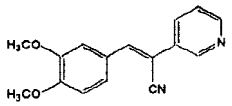
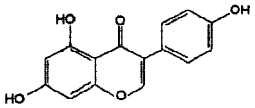
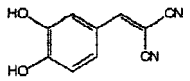
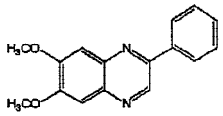
Compounds and Antibodies

Tyrphostins A1 [α-cyano-(4-methoxy)cinnamonitrile], A47 [α-cyano-(3,4-dihydroxy)thiocinnamide], B44(-) [α-cyano-(-)-(R)-N-(α-phenethyl)-3,4-dihydroxycinnamide], B48 [α-cyano-(3,4-dihydroxy)-N-phenylcinnamide], B50 [α-cyano-(+)-(S)-N-(α-phenethyl)-3,4-dihydroxycinnamide], AG1295 [(6,7-dimethyl-2-phenylquinoxaline)], RG13022 [α-(3′-pyridyl)-(3,4-dihydroxy) cinnamonitrile, and DAPI (4,6-diamino-2-phenylindole), and genistein were purchased from Calbiochem (La Jolla, CA). The chemical nature and some of the properties of these tyrphostins are presented in Table I. The tyrphostin stocks (10 to 25 mg/ml) were made in dimethylsulfoxide (DMSO) and diluted in culture medium immediately before adding to the cells. Control cultures were incubated in medium containing an equivalent amount of DMSO. Water-soluble 17β-estradiol, DMSO, DAB (3,3′-diaminobenzidine, and (3-[4,5-dimethyltiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). Mab Ki-67 was purchased from DAKO (Dako, Carpinteria, CA).

Cell Culture

MCF-7 cells were maintained in 10% FBS DMEM (GIBCO, Grand Island, NY) in a humidified 5% CO_2 atmosphere. Cultures were routinely screened for the presence of mycoplasma using DAPI and fluorescence microscopy. To achieve serum and phenol red starvation and relative quiescence, the culture medium was changed 24 hr after plating to 1% bovine serum albumin (BSA) in phenol red-free (PRF) DMEM. The cells were further cultured for 72 hr. Commonly, investigations of E2-stimulation of cell growth in vitro have used charcoal/dextran-stripped serum. However, such serum preparations are variable in their content of steroid hormones and

TABLE I. Typhostin-Inhibitory Constants*

Tyrphostin		Inhibition of MCF-7 cell growth[a]			EGFR-K polyGAT phosphorylation[b]	Selectivity
		E2-stimulated	Autonomous	Serum-stimulated		
A47		18	31	97	2.40	EGFR > bFGFR > PDGFR > InsR
B44(−)		Not inhibitory	Not inhibitory	41	2.50	EGFR > erbB-2 > InsR
B48		48	100	35	1.24	EGFR > erbB-2 > InsR
B50(+)		221	293	19	0.86	EGFR > erbB-2 > InsR
RG13022		168	73	220	1.00	EGFR > InsR
Genistein		Not inhibitory	Not inhibitory	385	2.6[c]	Broad, including ATP
A1		Not inhibitory	Not inhibitory	180	>1250	Inactive structural analog
AG1295		Not inhibitory	Not inhibitory	Not inhibitory	>100	PDGFR > InsR

*Values in μM.
[a]Inhibitory values determined from logarithmic curve fits of MTT assay data.
[b]IC (50) for the phosphorylation of polyGAT [4].
[c]IC (50) for EGFR kinase activity [33].

growth-promoting or growth-inhibiting substances [10]. In contrast, BSA medium provides uniform conditions.

Proliferation Assays

Proliferation of MCF-7 cells was measured in a 3-day MTT assay [11]. Cells were plated in FBS containing DMEM at a density of 10^4 cells/well in 96 well plates and allowed to adhere for 18–24 hr. Cultures were then washed once with PRF DMEM and fed with 1% BSA PRF DMEM. After 72 hr, the medium was aspirated and fresh medium containing dilutions of tyrphostins with or without E2 (200 pg/ml) was added and incubated for an additional 72 hr. The cultures were washed with PRF DMEM and treated with MTT diluted in the same medium (1 mg/ml, 50 μl) for 4 hr at 37°C. MTT solution was aspirated and the formazan crystals were dissolved in DMSO (150 μl) at 37°C for 2 hr. Relative cell numbers were determined based on the optical absorbance of the formazan at 570 nm using a control wavelength of 690 nm measured in an automatic plate reader (Molecular Dynamics, Sunnyvale, CA). Cell numbers in the control group, i.e., without tyrphostin treatment, were considered 100% and those in the treatment groups were calculated as percent of controls. Tyrphostins that reduced the cell number below that in the controls at less than 300 μM were considered inhibitory for cultures grown in 1% BSA or 10% serum containing media. To compare the efficacies of different tyrphostins, 25% inhibitory concentrations are calculated. For estimating the efficacy of tyrphostins against E2-induced stimulation, the concentrations of tyrphostins that completely block E2 stimulation are calculated. Those that were noninhibitory at greater than 300 μM were considered not effective.

Colony Formation Assay

Colony formation assay was performed essentially as described previously [12]. In brief, 10^3 MCF-7 cells were plated in 35-mm tissue culture dishes containing 10%

FBS DMEM. After 24 hr, the cultures were washed once with PRF DMEM and fed with 1% BSA in PRF DMEM. After 72 hr, the medium was replaced with fresh medium containing 0 to 400 μM of tyrphostin A47 with or without E2 (200 pg/ml). After 24 hr, the medium was removed and the cultures were washed twice with DMEM and fed with 10% FBS DMEM (1.5 ml). The medium was replenished every 3 or 4 days. After 12 days, the cultures were washed with PBS and fixed in methanol followed by giemsa staining. Colonies of 40 or more cells were counted under an inverted microscope. Numbers of colonies in the tyrphostin-treated groups expressed as percent of controls (cultures not treated with tyrphostin) are plotted as a function of tyrphostin concentration.

Ki-67 Staining

MCF-7 cells (5×10^4 cells) per coverslip were plated in 10% FBS DMEM and allowed to adhere in a humidified incubator at 37C. After 24 hr, the coverslips were washed once with 1-ml PRF DMEM and incubated in the same medium with 1% BSA. After 72 hr, the cells were treated with E2 and 45 or 135 μM of tyrphostin A47. After 48 or 72 hr of treatment, the cells were washed once with PBS and fixed in methanol. Ki-67 antigen was detected by immunostaining with a Ki-67 mAb (DAKO MO722), a mouse-specific peroxidase-conjugated secondary antibody and DAB. Cells were counterstained with Light Green SF Yellowish stain (Sigma), dehydrated, and the coverslips were mounted on slides. Ki-67–positive (brown nuclei or nucleoli) and –negative cells were counted under a microscope using a 40× objective.

RESULTS

Effect of Tyrphostins on the Growth of MCF-7 Cells

MCF-7 cells when maintained in medium without any exogenous growth promoting factors, i.e., in DMEM supplemented with 1% BSA, continued to proliferate at a minimal rate during the experimental duration (up to 6 days). This basal level of cell proliferation is ascribed to utilization of growth factors produced and/or secreted by the cells themselves (intracrine, autocrine, juxtacrine, and paracrine). MCF-7 cell proliferation increased in a dose-dependent manner when E2 was added to 1% BSA medium (Fig. 1). To test the effects of tyrphostins on autonomous proliferation of MCF-7 cells, the cells were first maintained in 1% BSA in the absence of phenol red, estrogen, or serum for 3 days, washed, and treated with 1% BSA medium containing different concentrations of tyrphostins. EGFR-selective tyrphostins, A47, B48, B50(+), and RG13022 reduced the cell numbers by at least 25% at concentrations of 31, 100, 293, and 73 μM, respectively (Fig. 2 and Table I). Tyrphostins B44(-), AG1295, A1, and genistein were ineffective as growth inhibitors. It is interesting to note that B50(+) was inhibitory, whereas its enantiomer B44(-) failed to growth-inhibit MCF-7 cells.

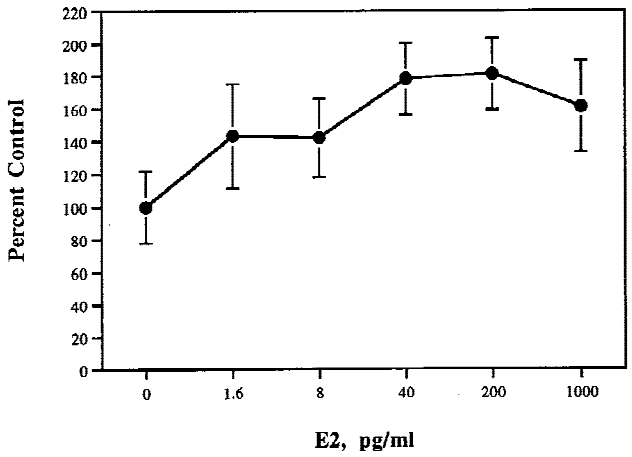


Fig. 1. Autonomous and E2-stimulated growth of MCF-7 cells in 1% BSA DMEM. Cells (10^4/well, 96 wells) were plated in 10% FBS DMEM and allowed to adhere for 18–24 hr. Cultures were washed once with PRF DMEM and 1% BSA PRF DMEM was added to each well. After 72 hr, an MTT assay was performed on one set of wells (day 0); for the remainder of the plate, the medium was aspirated and fresh medium containing 0–200 pg/mL of E2 was added. After 72 hr, cell number in each well was estimated by an MTT assay.

The effects of tyrphostins on E2-induced proliferation of MCF-7 cells were then determined in cultures that were first depleted of any residual estrogenic effects as described above. At this time, the cells were washed and the medium with or without estrogen but containing different concentrations of tyrphostins was added. Relative cell numbers were determined at 3 days by an MTT assay (Fig. 2). The purported EGFR-selective tyrphostins A47, B48, B50(+), and RG13022 inhibited the E2-stimulated growth of MCF-7 cells in a dose-dependent manner (Fig. 2). Complete inhibition of E2-mediated growth stimulation was achieved at 18, 48, 221, and 168 μM levels, respectively, by these tyrphostins (Table I). While B50(+) completely inhibited the E2-stimulated, the eanantiomer B44(-) was ineffective. The PDGF-selective tyrphostin AG1295 and genistein were not effective inhibitors of E2-stimulated proliferation of MCF-7 cells. Genistein, a known phytoestrogen, was in fact significantly growth-stimulatory for MCF-7 cells. Growth promoting effects of genistein on MCF-7 cells have been found by other investigators as well [13]. A1, the control RTK noninhibitory analog of tyrphostins, showed no inhibitory effects on E2-stimulated cell proliferation.

Figure 3 shows the pattern of inhibition of cell proliferation under the influence of serum factors. All EGFR-selective tyrphostins, namely, A47, B44(-), B48, B50(+), and RG13022, were growth-inhibitory to serum-stimulated MCF-7 cells. The 25% inhibitory concentrations were 97, 41, 35, 19, and 220 μM, respectively.

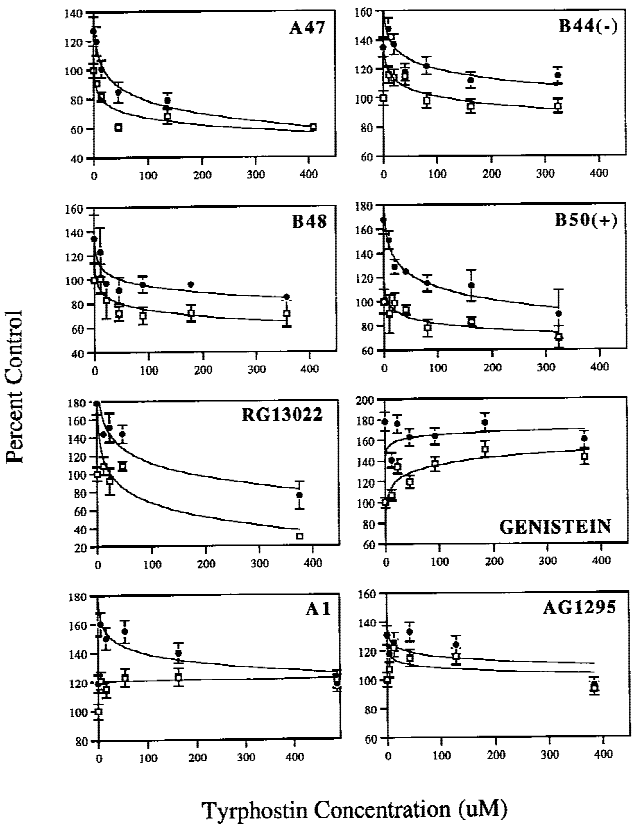


Fig. 2. Effect of tyrphostins on autonomous and E2-stimulated growth of MCF-7 cells determined by an MTT assay. A total of 10^4 MCF-7 cells/well were seeded in 10% FBS medium in 96 well plates. After 18–24 hr, cells were subjected to serum and phenol red starvation for 72 hr. The cells were then exposed to different concentrations of tyrphostins and 0 or 200 pg/mL estrogen for 3 days. Cell numbers in the untreated control and treatment groups were estimated by an MTT assay. Cell numbers as percent of controls were plotted as a function of tyrphostin concentration and the curves were fit with the standard logarithmic algorithm, $y = a0 \log(X) + a1$.

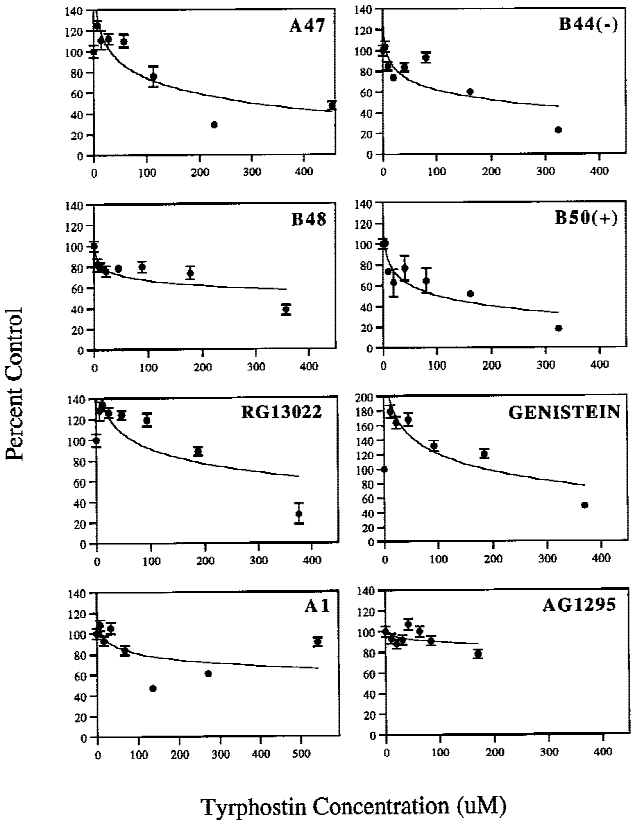


Fig. 3. Effect of tyrphostins on the serum-stimulated growth of MCF-7 cells in vitro. Cells (10^4/well, 96 wells) were plated in 10% FBS DMEM and allowed to adhere for 36 hr. The medium was aspirated and fresh medium containing varying concentrations of tyrphostsins was added. After 72 hr, the relative number of cells was assessed by an MTT assay. Cell numbers in the untreated control and treatment groups were estimated by an MTT assay. Cell numbers as percent of controls were plotted as a function of tyrphostin concentration and the curves were fit with the standard logarithmic algorithm, $y = a0 \log(X) + a1$.

Genistein, AG1295, and A1 showed little or no inhibitory effects. Genistein at low concentrations was growth-stimulatory in the presence of serum, consistent with its phytoestrogenic nature. In the presence of serum, the inhibitory effects of different tyrphostins differed from those in the absence of growth stimulants or in the presence of E2. This difference is likely because the panel of tyrphostins we tested are predominantly EGFR-selective and serum contributes a variety of additional growth stimuli. Thus, while the EGFR-selective tyrphostins block cell proliferation, proliferation induced by other growth factors in the serum is either not inhibited or inhibited to a lesser degree.

Since we tested the antiproliferative effects of a number of tyrphostins on a single cell line, we could examine any correlation between structure/function of tyrphostins and their growth-inhibitory effects. As an example, we looked at the reported EGFR-inhibitory constants for tyrphostins obtained by following the phosphorylation of an exogenous tyrosine kinase substrate, polyGAT [4], and their growth-inhibitory effects. Those tyrphostins that inhibited the EGFR tyrosine kinase were generally growth-inhibitory to E2-stimulated cells, consistent with the findings that EGF is an important mediator of E2-induced growth stimulation. However, correlation between the IC_{50}s for inhibition of polyGAT phosphorylation and the degree of inhibition of E2-stimulated growth of MCF-7 cells was not strong. Such a lack of correlation may be due to differences in the stability of the compounds, ability to enter the cells, and potential modifying factors within the cells.

Effect of Tyrphostin A47 on Colony Formation

In order to ensure that our MTT results are a true representation of viable cell number and cell proliferation but not the result of inhibition of mitochondrial function, a colony formation assay was performed. In this assay, MCF-7 cells were briefly exposed to increasing

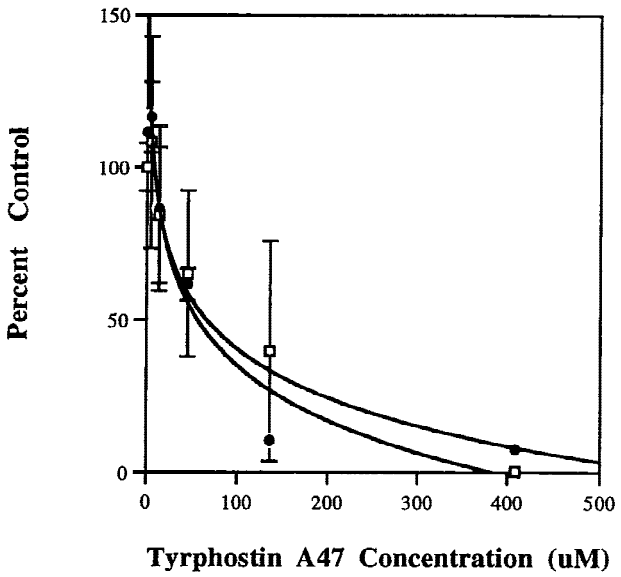


Fig. 4. The effect of tyrphostin A47 and E2 on MCF-7 colony formation. MCF-7 cells (1,000 cells, 35-mm tissue culture dish) were plated in 10% FBS DMEM. After 24 hr, the cultures were subjected to serum and phenol red starvation. After 72 hr, the medium was aspirated and fresh medium with 0–300-μM tyrphostin A47 and with or without 200-pg/mL E2 was added. After 24-hr incubation, the cultures were washed and fed with 10% FBS DMEM. Medium was replenished every 3 to 4 days. After 12 days, the cultures were washed with PBS, fixed in methanol, and stained with giemsa. Colonies of 40 cells or more were counted with the aid of an inverted microscope. The numbers of colonies were plotted as a function of tyrphostin concentration. Open circles: cells treated in the absence of E2; filled circles: cells treated with 200-pg/mL estrogen.

amounts of a representative tyrphostin (A47) in the presence or absence of E2 and the number of colonies formed after 12 days was assessed. As was noted in the MTT assay, proliferation of MCF-7 cells was markedly higher in the presence of E2 than in its absence, thereby the number of colonies/dish in the presence of E2 reached about 800, whereas in the absence of E2 it was about 200. A47 blocked colony formation both in the absence and presence of E2 in a linear dose-dependent manner, with complete inhibition of colony formation above 100 μM (Fig. 4). Inhibition of colony formation expressed as percent of control is similar in the E2-treated and -untreated groups. This result confirms that cell proliferation is inhibited by the tyrphostin A47 and that the results from MTT assay are not due to inhibition of mitochondrial enzymes by tyrphostins. Furthermore, it appears that a short treatment (24 hr) exerts a long-lasting effect on cell proliferation.

Effect of Tyrphostin A47 on Cell Cycle Progression

As a further test to validate the MTT assay results, the effects of tyrphostin A47 on the entry of MCF-7 cells into cell cycle was investigated by Ki-67 immunostaining. The Ki-67 antigen is a nuclear-specific protein expressed in all proliferating cells in late G1, S, M, and G2 phases [14]. Cells entering the mitotic cycle in cultures maintained in 1% BSA was approximately 6% at both 48 and 72 hr. Cells entering the cell cycle increased to 26%–34% when they were grown in medium containing serum or 200 pg/mL estrogen. Percent MCF-7 cells positive for Ki-67 in cultures treated with either 45-μM A47 and E2 were 17 and 7, respectively, at 48 hr. There was a further reduction to 5% and 3% when the cells were treated with 135-μM A47. Thus, by 72 hr, percent cycling cells in cultures treated with E2 and A47 was less than that seen with cultures in 1% BSA (Table II). These results further demonstrate the validity of MTT assay as a measure of cell proliferative activity.

TABLE II. Effects of E2 and Tyrphostin A47 on the Expression of Ki-67 Antigen in MCF-7 Cells*

Treatment group	48 hr	72 hr
FBS	26.6 ± 4.2	31.8 ± 8.3
BSA	6.6 ± 2.4	5.9 ± 4.1
BSA + 200-pg/ml E2	26.1 ± 6.1	34.1 ± 9.8
BSA + 200-pg/ml E2 + 45-μM A47	16.9 ± 4.3	4.9 ± 2.5
BSA + 200-pg/ml E2 + 135-μM A47	7.2 ± 2.7	2.7 ± 2.0
BSA + 45-μM A47	12.4 ± 3.6	18.7 ± 5.4
BSA + 135-μM A47	6.8 ± 2.7	2.4 ± 1.5

*Data as percent total cells. MCF-7 cells (5,000 in 1 ml) were plated on coverslips in 10% FBS DMEM and allowed to adhere in a humidified incubator at 37°C. After 24 hr, the coverslips were washed once with 1-ml PRF DMEM and incubated in DMEM with 1% BSA. After 72 hr, the cells were treated with E2 and 45-μM or 137-μM of tyrphostin A47. After 48 or 72 hr of treatment, the cells were washed once with PBS and alcohol-fixed. Ki-67 antigen was detected by immunostaining with a Ki-67 mAb (Dako), a mouse-specific peroxidase-conjugated second antibody and DAB. Cells were counterstained Light Green, dehydrated, and the coverslips were mounted on slides. Ki-67–positive cells were counted under a microscope using a 40× objective.

DISCUSSION

Tyrphostins represent an interesting family of small-molecular-weight compounds that inhibit activation of receptor tyrosine kinases. Since activation of RTKs is a critical step in growth factor-induced cell proliferation, it is hoped that tyrphostins may serve as important agents for controlling the growth of neoplastic cells. Furthermore, since different members of the tyrphostin family selectively act on different growth factor receptors, they serve as useful tools to illuminate on the role of different growth factors in proliferation of cells under different conditions. In this regard, our current interest has been to identify tyrphostins that can block estrogen-mediated growth stimulation of hormone-dependent breast cancer cells. This interest has relevance to treatment of postmenopausal breast cancer survivors receiving hormone replacement therapy (HRT). It is well appreciated that HRT has several beneficial effects on postmenopausal women, including reduction of cardiovascular disease, osteoporosis, colon cancer, and Alzheimer disease [15–

19]. However, it is not clear whether HRT can be given to breast cancer survivors as it may enhance the growth of any residual microscopic disease [20–22]. Since at least some of the estrogenic growth stimulation is growth factor-mediated [23], receptor tyrosine kinase inhibitors may serve as adjuncts to mitigate the potential tumor growth-stimulatory effects of E2 and make HRT safer to postmenopausal breast cancer survivors. With this goal in mind, we have currently investigated a panel of tyrphostins for their antiproliferative effects on hormone-dependent human breast cancer cell line, MCF-7, in vitro.

Our results show that tyrphostins can effectively inhibit E2-induced proliferation of breast cancer in vitro. This was particularly notable with the EGFR-selective tyrphostins implying that the mitogenic signal from E2 is mediated by an EGFR-related tyrosine kinase. Furthermore, the selectivity shown by our panel of tyrphostins toward growth inhibition of MCF-7 cells without any exogenous growth factor also suggests that under serum-free conditions, autocrine EGF/TGFα may have an important role in MCF-7 growth stimulation. This thought is supported by findings from several investigators. Anti-EGFR antibodies have been found to block the autonomous proliferation of MCF-7 as well as proliferation induced by exogenous EGF [24,25]. Tyrphostin RG13022 and A47 have both been shown to inhibit EGF-induced autophosphorylation of the EGFR [6,26]. The inhibition of EGFR phosphorylation in both cases was shown to correlate with the inhibition of EGF-stimulated cell proliferation. Incidentally, neither tyrphostin had any effect on receptor ligand binding activity, biosynthesis, or turnover of the EGFR.

EGF has been shown at least to replace partially the requirement for estrogen to stimulate hormone-dependent experimental tumors in vitro and in vivo [27,28]. Reddy et al. [6] have also examined the effect of the EGFR-selective tyrphostin RG13022 on E2-stimulated growth of MCF-7 cells in vitro. RG13022 was found to block totally E2-stimulated proliferation of MCF-7 cells. These results suggest that EGF may be an important growth factor recruited by E2 to induce proliferation of MCF-7 cells and that blockade of EGFR phosphorylation may be used to growth-inhibit effectively MCF-7 cell line. Accordingly, the EGFR-selective tyrphostins, A47, B48, B50(+), and RG13022 block the E2-stimulated growth of MCF-7 cells in an MTT proliferation assay.

Other investigators have reported that the benzylidene malononitrile-based tyrphostin, AG17, uncouples oxidative phosphorylation in a wide variety of cells [29]. Our MTT assay depends on mitochondrial enzyme reduction of tetrazolium dye to determine the viable cell numbers. In order to ensure that our MTT assay results are a true representation of the cell numbers, we performed a colony formation assay and Ki-67 immunostaining method. Tyrphostin A47 was found to inhibit the E2-induced MCF-7 cell entry into cell cycle in a dose-dependent manner. This finding not only confirms the validity of MTT assay but also is consistent with blockade of the growth factor receptor-mediated initiation of cell division by A47.

Tyrphostins used in this study have mainly been defined for their ability to inhibit selectively the phosphorylation of the EGFR peptide substrate, polyGAT [30]. Although the growth-inhibitory capacity of a tyrphostin may have been extended to correlate with its ability to inhibit the autophosphorylation of EGFR, there may be other structurally related tyrosine kinases that function in other pathways of cell division that could be affected. Faaland et al. [8] found a 90% reduction in cyclin B1 in A47-treated MCF-7 cells and suggested that the inhibition of proliferation by A47 occurred as a result of mechanisms other than those involving EGFR signaling. While these studies contribute to an understanding of the physiological effects of tyrphostins, important findings are that A47 inhibits the E2-mediated growth of cancer cells in vitro as well as MCF-7 xenografts in vivo [9] at nontoxic doses. We have further found in our preliminary studies that at tumor growth-inhibitory doses, A47 does not affect the formation of bone nodules by osteoblasts in vitro [31,32]. Thus, it appears that growth factor receptor kinase inhibitors may be useful to block the potential tumor growth-stimulatory effects of E2 in postmenopausal breast cancer survivors receiving hormone replacement therapy.

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